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# Sewage from airplanes exhibits high abundance and diversity of antibiotic resistance genes

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## 1 Abstract

2 Airplane sanitary facilities are shared by an international audience. We hypothesized  
3 the corresponding sewage to be an extraordinary source of antibiotic resistant bacteria  
4 (ARB) and resistance genes (ARG) in terms of diversity and quantity. Accordingly,  
5 we analyzed ARG and ARB in airplane-borne sewage using complementary approaches:  
6 metagenomics, quantitative PCR, and cultivation. For the purpose of comparison, we also  
7 quantified ARG and ARB in the inlets of municipal treatment plants with and without  
8 connection to airports. As expected, airplane sewage contained an extraordinary rich  
9 set of mobile ARG and the genes' relative abundances were mostly increased compared  
10 to typical raw sewage of municipal origin. Moreover, combined resistance against third  
11 generation cephalosporins, fluoroquinolones and aminoglycosides was unusually common  
12 (28.9%) among *E. coli* isolated from airplane sewage. This percentage exceeds the one  
13 reported for German clinical isolates by a factor of eight. Our findings suggest that  
14 airplane-borne sewage can effectively contribute to the fast and global spread of antibiotic  
15 resistance.

16 **Keywords:** antibiotic resistance, airplane, sewage, wastewater, diversity

# 1 Introduction

Since their discovery in the 1940s, antibiotics saved millions of lives, but due to the global spread of resistance genes these drugs rapidly lose their activity. This threatens the very core of modern medicine by limiting the means to effectively cure bacterial infections ([World Health Organization, 2015](#)). In the era of globalization and high mobility, pathogenic strains carrying antibiotic resistance genes (ARG) are spreading quickly and globally ([Nordmann et al., 2011](#)). In the endeavor to decelerate or stop the spread of antibiotic resistance, it is necessary to identify the hotspots and pathways of ARG dissemination.

Airports are places where sewage of people from different parts of the world is collected and, after treatment, released in the local aquatic environment. It is thus reasonable to assume that airports serve as an entrance for ARG which are endemic in specific parts of the world while being rare or absent in the flights' country of destination. This should be reflected in a particularly high diversity of ARG in airplane-borne sewage as compared to conventional municipal wastewater (**hypothesis 1**).

The prevalence of antibiotic resistant bacteria (ARB) is known to be linked with veterinary and human antibiotic use ([World Health Organization, 2014](#)). Specifically, [Forsslund et al. \(2013\)](#) found the resistance potential in human guts to be positively correlated with country-specific antibiotic use. In Germany, antibiotic consumption is relatively low compared to, e.g., China, India, or the USA ([Van Boeckel et al., 2014](#)) and it is also lower than in many member states of the EU ([European Surveillance of Antimicrobial Consumption Network, 2017](#)). Consequently, airplane-borne sewage was hypothesized to contain ARB and ARG in elevated abundances as compared to standard municipal sewage generated by the local population (**hypothesis 2**).

The two hypotheses related to the diversity and abundance of ARG were tested by the complementary approaches of metagenome sequencing, quantitative PCR (qPCR), and cultivation, thereby providing a comprehensive picture of antimicrobial resistance in studied systems. Specifically, the strength of the metagenomics approach lies in its capability to detect and quantify the full spectrum of ARG with the downside of limited sensitivity. Quantitative PCR, on the other hand, allows even rare genes and gene variants to be quantified since the target sequences are specifically amplified. However, qPCR necessarily has a narrow focus dictated by the chosen primers. Finally, susceptibility tests provide the only means to study phenotypic antibiotic resistance, including multi-resistance. Unlike the other techniques, cultivation covers a small part of the microbial community only.

In this study, any of the three approaches was used to quantify antibiotic resistance in untreated sewage collected from (1) airplane tanks and (2) the inlets of municipal wastewater treatment plants (WWTP). The set of WWTP was chosen such that some of the plants received wastewater from the nearest airport while others did not. The latter distinction was made to test whether the hypothesized peculiarities of airplane-borne sewage are still noticeable after mixing with "conventional" municipal wastewater.

## 2 Material and methods

### 2.1 Sampling

Eight grab samples of airplane-borne sewage were obtained from five different airports, three of which were sampled twice (with sufficient delay to ensure independence). Three of the airports had >20 million passengers p.a. in 2015 while the other two were of intermediate size (>10 million passengers p.a.) or smaller. The sewage was gathered from vacuum trucks collecting the waste from multiple aircrafts. Since the aircraft's

65 tanks are emptied on demand, each tank contains sewage from multiple flights. The  
66 samples thus represent pooled samples integrating over space and time.

67 Incoming sewage from WWTP having a connection to airports was collected at six  
68 different locations, two of which were visited twice (total number of eight samples; each  
69 being a 24 h composite). At the respective plants, airport-borne sewage was known  
70 to contribute at least 20% of the total inflow. These 20% represent a mixture of fecal  
71 material from airplane tanks with sewage from other airport facilities such as terminal  
72 buildings. Detailed data on mixing ratios and its temporal variation were unavailable to  
73 us. Surface runoff from airports is generally disposed separately from sewage not least  
74 because of the possible contamination with, e.g., de-icing agents.

75 Incoming sewage from WWTP without connection to airports was collected at six  
76 locations. At one of the locations, samples were taken from four different sewers and  
77 another plant was sampled multiple times (with sufficient delay to ensure independence).  
78 The total number of 24 h composite samples was 19.

79 All samples were stored in 1 L sterile glass bottles at 4 °C and processed within 24 h.  
80 Relevant meta-information is provided in Table S.1.

## 81 2.2 Metagenomics

82 DNA was extracted using the PowerWaterKit (MoBio, Vancouver, Canada; see Ta-  
83 ble S.1 for amount and quality of DNA). All samples were shotgun-sequenced on a  
84 MiSeq device (2×150 bp; GATC Biotech AG, Konstanz, Germany). Quality checking and  
85 trimming (q=28, minimum length=100 bp) was performed using TrimGalore! ([http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)). The preprocessed  
86 forward reads were then aligned to the latest (2019-03-05) **resfinder** data base (Zankari  
87 et al., 2012). Specifically, we relied on the python implementation of the **resfinder**  
88 script which is publicly available from the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org/>). The **resfinder** script attempts to match the nu-  
89 cleotide sequences contained in the samples with the sequences of known ARG as stored in  
90 the **resfinder** data base. Internally, the **resfinder** script calls the Basic Local Align-  
91 ment Search Tool (**blastn**; <http://blast.ncbi.nlm.nih.gov>) to compare nucleotide  
92 sequences. **blastn**-reported alignments with e-values exceeding  $10^{-10}$  were generally  
93 ignored to minimize the chance of false positive hits.

94 The set of unique ARG contained in a particular sample was established from the full  
95 list of resistance genes whose signatures matched with a nucleotide sequence from the  
96 sample. Technically speaking, the obtained list of hits was filtered to remove any dupli-  
97 cate gene identifiers. The **resfinder** data base holds information on a large number genes  
98 and variants, e.g. about 2000 of the registered ARG target beta-lactams. So as to focus  
99 on ARG diversity at a higher level, we generally truncated the genes' identifiers at the  
100 first underscore before establishing the unique set of ARG. For example, instead of count-  
101 ing the tetracycline resistance gene variants **tet(M)\_7\_FN433596** and **tet(M)\_1\_X92947**  
102 as individual instances, they were commonly registered as just **tet(M)**. For the purpose  
103 of additional quality assurance, a particular gene was only registered as present if its  
104 signature was detected at least three times. Moreover, nucleotide sequences matching  
105 the signatures of multiple ARG registered in the **resfinder** data base (ambiguous hits),  
106 were ignored altogether.

## 109 2.3 Quantitative PCR

110 Fourteen ARG were selected for qPCR-based quantification in line with activities of  
111 international research consortia investigating antimicrobial resistance in aquatic environ-  
112 ments. The set of ARG includes common and rare ones with a special focus on clinically

relevant genes coding for carbapenemases (*bla*<sub>TEM</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>CTX-M-32</sub>, *bla*<sub>CMY-2</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>OXA-58</sub>, *bla*<sub>KPC-3</sub>, *bla*<sub>NDM-1</sub>, *bla*<sub>VIM-2</sub>, *ermB*, *mecA*, *mcr1*, *tetM*, *sul1*). All ARG as well as 16S rRNA genes were quantified following the same procedure as described in Heß et al. (2018). The relevant references for the primers of the additionally quantified ARG are the NORMAN network ([www.norman-network.net](http://www.norman-network.net)) for *bla*<sub>TEM</sub>, and *bla*<sub>CTX-M-32</sub>, the ANSWER project ([www.answer-itn.eu](http://www.answer-itn.eu)) for *bla*<sub>CTX-M-15</sub>, Kurpiel and Hanson (2012) for *bla*<sub>CMY-2</sub>, Monteiro et al. (2012) for *bla*<sub>OXA-48</sub>, Hembach et al. (2017) for *mcr1*, and Peak et al. (2007) for *tetM*. Primer sequences can be found in Table S.2. For the 16S rRNA gene, *sul1* and *bla*<sub>CTX-M-32</sub> the pNORM plasmid designed by Ch. Merlin (University of Lorraine, France; [www.norman-network.net](http://www.norman-network.net)) was used as standard. For the remaining genes, standards were created by cloning the respective qPCR amplicons into the pGEM-T vector (Promega, Madison, Wisconsin, USA).

The detection limit was  $10^2$  copies per reaction for all genes. The efficiency of these assays was between 0.9 and 1 with  $R^2 > 0.997$  for all the runs. All genes were quantified in duplicates with a standard deviation of the cycles  $< 0.2$ .

## 2.4 Standardization of data and community analysis

In the context of this study, the diversity and abundance of ARG was to be compared across samples of varying composition and dilution (toilet waste, municipal wastewater), hence, standardization was required. With regard to ARG abundances, we adopted the common practice of dividing absolute ARG counts by the corresponding number of 16S rRNA copies (see, e.g. Pärnänen et al., 2019). The resulting numbers are referred to as *relative abundances*. We applied the same standardization also to metagenomics-based information on ARG diversity. Specifically, the diversity of resistance genes was expressed as the number of unique ARG per 1000 copies of 16S rRNA genes.

The calculation of relative ARG abundances from qPCR-based data involved a qPCR-based quantification of 16S rRNA genes (see Table S.2 for primer). Likewise, metagenomics-based information on ARG was standardized using metagenomics-based estimates on the abundance of 16S rRNA genes. The latter was extracted from the nucleotide sequences using METAXA2 (Bengtsson-Palme et al., 2015b, version 2.1.3).

The number of 16S rRNA gene copies per bacterial cell is known to vary between taxonomic groups. Consequently, a comparison of relative ARG abundances (or ARG diversities) requires that the respective microbial communities are similar in terms of taxonomic composition or, at least, with regard to the community-weighted average number of 16S rRNA gene copies per cell. We employed METAXA2 to infer information on the composition of the microbial community based on 16S rRNA. Finally, we relied on the rrnDB data base (Stoddard et al., 2015) to compute for all metagenomics-samples the expected average number of 16S rRNA copies per bacterial cell taking into account taxonomic groups and their proportions. The matching of taxonomic items reported by METAXA2 with items registered in rrnDB was successful at family level in 86% of the cases, at genus level in 54% of the cases and at species level in 10% of the cases.

## 2.5 Bacteria isolation and susceptibility testing

*Escherichia coli* was chosen as a model organism because it is widely considered as a fecal indicator and resistance levels of *E. coli* from different origins are well documented (e.g. European Centre for Disease Prevention and Control, 2017; Osifinka et al., 2017; Rosas et al., 2015). *E. coli* is furthermore a potential pathogen and its harboring of resistance genes can thus directly impact human health.

To obtain at least 24 *E. coli* isolates from each sample, suitable dilutions were plated on mFC agar (Carl Roth, Karlsruhe, Germany). After  $18 \pm 2$  h of incubation at 44 °C

161 blue colonies were streaked on Brilliance agar (Oxoid, Wesel, Germany) and grown  
 162 overnight at 37 °C to obtain pure cultures. To identify the isolates as *E. coli*, colony  
 163 PCR was performed as in [Heß et al. \(2018\)](#) to amplify a species specific fragment of the  
 164 *yccT* gene.

165 In total, 1140 *E. coli* isolates (Table S.1) were tested against 24 antibiotics which are  
 166 commonly used to treat the respective infections (187 isolates from airplanes, 161 and  
 167 362 isolates from the inflow of municipal treatment plants with and without connection  
 168 to an airport). The tests followed the EUCAST guidelines (agar diffusion test; [www.  
 169 eucast.org](http://www.eucast.org)) with *Escherichia coli* ATCC 25922 as a quality control. Applying the clinical  
 170 breakpoints defined by EUCAST, the isolates were classified as resistant or susceptible  
 171 (intermediary was counted as susceptible).

## 172 2.6 Statistics

173 Data analysis was conducted with R 3.4.3 ([R Core Team, 2017](#)). Relative ARG abun-  
 174 dances in samples of different origin were compared using the Welch test (`t.test()`)  
 175 with log-transformed data. A non-parametric rank sum test (`wilcox.test()`) was used  
 176 to test for differences in ARG richness. Proportion data were tested for significant dif-  
 177 ferences using Fisher’s exact test (`fisher.test()`). In case of multiple tests, p-values  
 178 were conservatively adjusted with the default `p.adjust()` method [Holm \(1979\)](#). p-values  
 179 were marked with asterisks according to the usual convention where \* indicates  $p \leq 0.05$ ,  
 180 \*\* denotes  $p \leq 0.01$  and \*\*\* corresponds to  $p \leq 0.001$ . Bootstrap confidence inter-  
 181 vals (Fig. 5) were generated with `boot()` using ordinary resampling and  $10^4$  replicates.  
 182 Rarefaction curves (Fig. 3) were constructed from metagenomics data by evaluating an  
 183 increasing number of sequences from the whole set of sequences available for a particular  
 184 sample by means of sampling without replacement (R method `sample`). To minimize  
 185 random effects, we considered the medians of five replicate rarefaction curves per sam-  
 186 ple. Statistical models fitted to empirical rarefaction curves (Fig. 3) take the structure  
 187 of Eqn. 1 where  $R$  represents the number of different ARG,  $n$  denotes the number of  
 188 analyzed sequences, and  $R_{inf}$  and  $h$  are free parameters, respectively, fitted with R’s  
 189 default optimizer (`optim`).

$$R = R_{inf} \cdot \left( \frac{n}{n + h} \right)^{0.5} \quad (1)$$

190 Based on the overall number of sequences in a sample and the corresponding num-  
 191 ber of 16S rRNA copies, the variable  $n$  was adjusted individually for each sample to  
 192 pragmatically compensate for varying proportions of bacterial and non-bacterial DNA.

## 193 3 Results

### 194 3.1 Characterization of samples

195 In accordance with expectation, untreated wastewater sampled at WWTP inlets was  
 196 generally more diluted as compared to the sewage derived from airplane tanks. This is  
 197 reflected, for example, in the electric conductivity but also in the number of 16S rRNA  
 198 gene copies per volume (Table 1). In addition, airplane sewage exhibited higher values  
 199 of pH which is likely due to an elevated concentration of soap residues.

200 The metagenomics-derived information on 16S rRNA fragments allowed for a deeper  
 201 characterization of the samples in terms of the composition of the bacterial community.  
 202 The latter was very similar in all samples taken at the inlets of WWTP whereas airplane-  
 203 borne samples showed larger variations in the contribution of different bacterial orders  
 204 (Fig. 1). Moderate contrasts were observed between samples of different origins in terms

Table 1: Mean values of electric conductivity (EC), pH, and the concentration of 16S rRNA genes in samples of different origins. The corresponding information for individual samples can be found in Table S.1.

Origin	EC (mS cm <sup>-1</sup> )	pH	16S rRNA gene copies mL <sup>-1</sup>
Airplane	10.1	8.9	$1.6 \times 10^9$
WWTP, with airport	1.7	7.3	$2.6 \times 10^8$
WWTP, no airport	1.1	7.3	$2.9 \times 10^8$

of taxonomic diversity. For example, the signatures of 6.5 bacterial families (median) were detected in  $10^5$  nucleotide sequences obtained from airplane sewage. This compares to values of 4 and 3 for wastewater from WWTP with and without connection to airports, respectively. This fact is also visible in Fig. 1 which shows a greater evenness in the contribution of taxonomic groups for airplane-borne samples as compared to WWTP samples. Characteristic differences between samples of different origins were observed, e.g., for Aeromonadales and Campylobacteriales (rare in airplane sewage but common in WWTP samples) as well as Enterobacteriales and Erysipelotrichiales (rare in WWTP samples but highly abundant in some airplane samples).

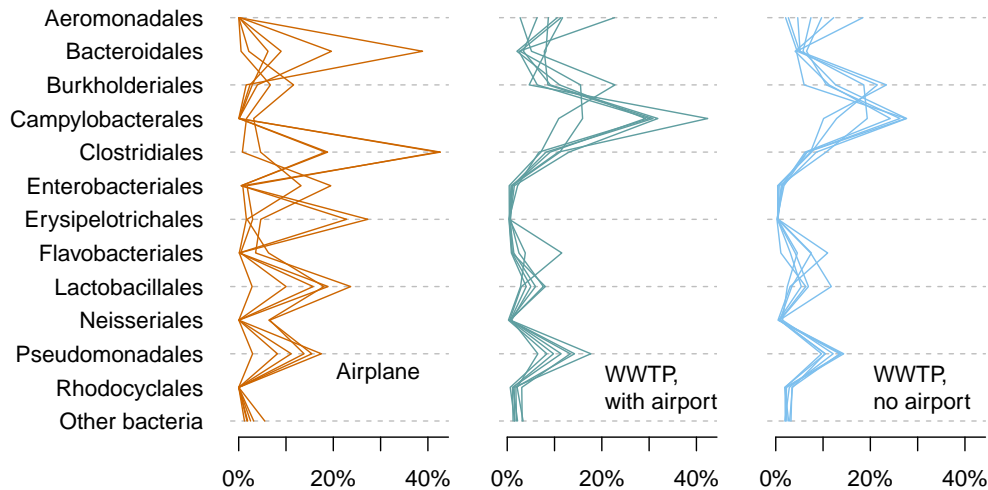


Figure 1: Percentage of 16S rRNA fragments attributable to different bacterial orders. Lines depict individual samples. Bacterial orders which did not contribute at least 5% in any of the samples were dropped for the sake of clarity.

In spite of the differences in taxonomic composition, the expected number of 16S rRNA genes per cell varied only little between sample of different origin. The merging of the metagenomics-based taxonomy data with information from the *rrnDB* data base resulted in a mean value of 4.9 copies of 16S rRNA genes per cell for airplane-borne samples. The respective mean values for raw wastewater with and without connection to airports were 4.5 and 4.8, respectively. The most extreme ratios observed for individual samples ranged between 4 and 5.5 and none of the differences in means fulfilled the criteria of statistical significance (ANOVA:  $p > 0.23$ ; all  $p > 0.13$  in post-hoc rank sum tests). In view of that, a standardization of ARG counts by the corresponding number of 16S rRNA genes (see Sec. 2.4) was considered appropriate. In other words, it is very unlikely that marked shifts in the relative abundance of ARG, e.g. between samples of different origin, merely reflect dissimilarities of the respective bacterial communities.



### 3.2 Diversity of antibiotic resistance genes

As outlined in Sec. 2.2, the diversity of antibiotic resistance genes was expressed as the number of unique ARG per 1000 copies of 16S rRNA genes. That measure of diversity was found to be significantly increased in airplane sewage as compared to untreated sewage sampled from the inlets of municipal WWTP (Fig. 2;  $p < 0.05$ , Wilcoxon rank sum test). On average, a unique ARG was detected every 580 copies of 16S rRNA genes in airplane sewage. In the WWTP samples, a unique ARG appeared every 750 copies of 16S rRNA genes. WWTP samples were statistically similar in terms of ARG diversity regardless of whether the plant received sewage from an airport or not (blue vs. green boxes in Fig. 2).

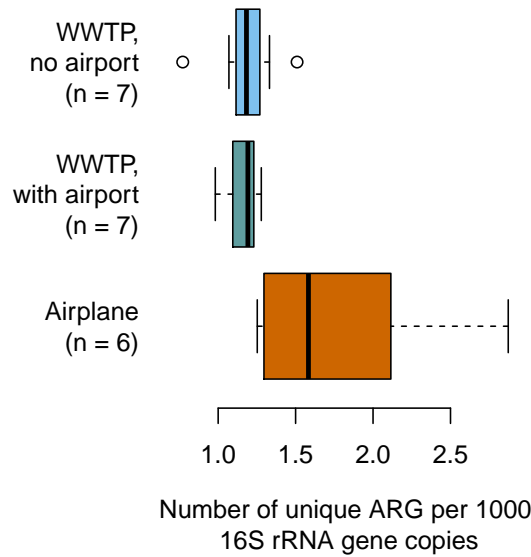


Figure 2: Diversity of antibiotic resistance genes in samples of untreated sewage collected from airplanes and WWTP with/without connection to airports. Whiskers extend to the most extreme data points not being classified as outliers.

The numbers on ARG diversity presented in Fig. 2 represent point estimates based on the number of sequences in each sample and the respective number of 16S rRNA gene copies. In order to verify the robustness of these estimates, we computed rarefaction curves for all samples processed with metagenomics (Fig. 3). The latter confirm that samples of airplane sewage are likely to contain a greater number different ARG in a given amount of DNA as compared to untreated wastewater. For example, the average number of unique ARG per  $5 \times 10^6$  sequences was about 110 in airplane-borne samples compared to about 85 in samples taken from the inlets of WWTP (Fig. 3).



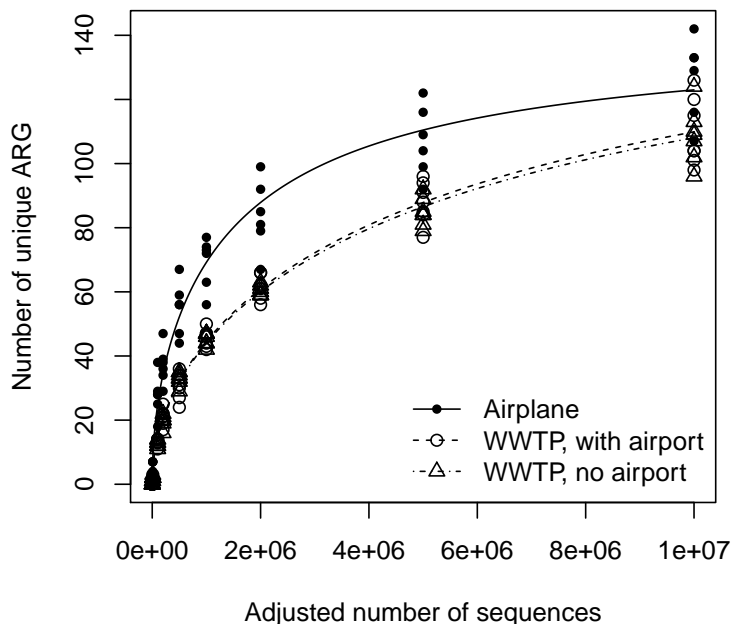


Figure 3: Rarefaction curves relating the number of unique ARG to the number of analyzed sequences (adjusted for varying abundances of 16S rRNA genes). Point symbols represent empirical estimates for individual samples. Statistical models of the form of Eqn. 1 (lines) were fitted to all points of a particular sample origin.

### 3.3 Abundance of antibiotic resistance genes

The metagenomics data indicate an increased relative abundance of ARG in airplane sewage as compared to sewage sampled at the inlets of WWTP (Fig. 4). The elevated prevalence in airplane-borne sewage was most pronounced for ARG directed against phenicols, sulphonamides, and tetracyclin ( $p < 0.01$ ) as well as aminoglycosides and macrolides ( $p < 0.05$ , two-sided Welch test, conservatively adjusted for multiple testing). Contrary to the trend, ARG targeting colistin were more abundant in municipal sewage than in airplane sewage (value near limit of quantification). Generally, the samples taken at treatment plants with and without connection to airports (green and blue symbols in Fig. 4) did not exhibit significant differences in terms of relative abundances according to the Welch test.

The auxiliary qPCR-based analyses support the main outcome of the metagenomics approach depicted in Fig. 4. Specifically, the relative abundances of *sul1* (sulphonamide) and *tetM* (tetracycline) resistance genes were significantly increased in airplane samples compared to samples taken at the inlets of WWTP (Table 2). The median relative abundances differ between the two sample origins by factors of 5 (*sul1*) and 18 (*tetM*), respectively.

With the exception of *bla<sub>VIM-2</sub>*, differences in relative abundance between airplane and WWTP samples were not statistical significant for the remaining ARG (Table 2). Especially for the  $\beta$ -lactamase genes, this finding is compatible with the metagenomic results (Fig. 4) according to which the total prevalence of genotypic  $\beta$ -lactam resistance was only slightly increased in airplane-borne samples.

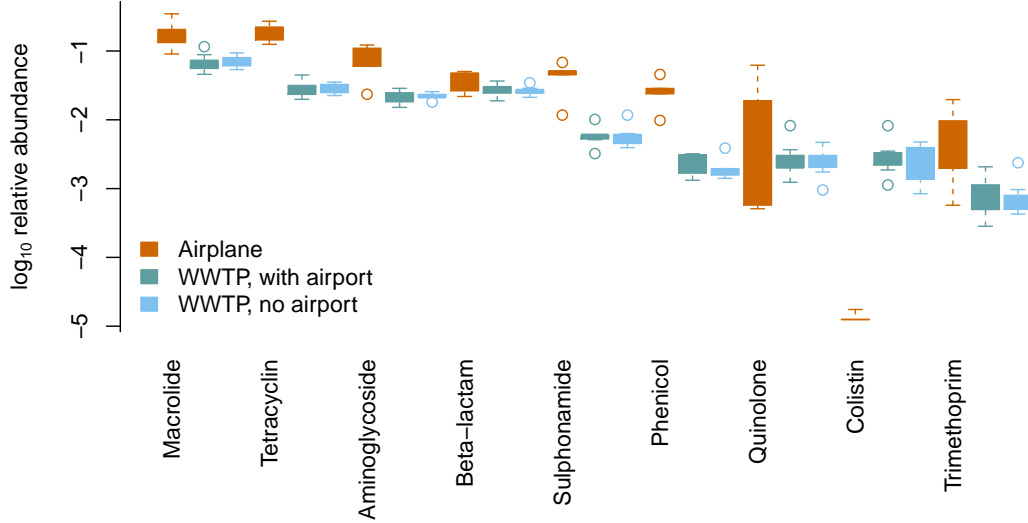


Figure 4: ARG copies per 16S rRNA gene copies in untreated sewage collected from airplanes and WWTP with/without connection to airports. The respective information on ARG and 16S rRNA genes is based on the metagenomics analysis. Genes were aggregated by target drug classes to reduce the complexity of information.

266 WWTP samples with and without contribution of airport sewage were also processed  
 267 through qPCR. Like with the metagenomics approach, statistically significant differences  
 268 between the two origins in terms of relative abundance could not be established (same  
 269 set of ARG as in Table 2, results not shown).

Table 2: Relative abundance of resistance genes (ARG copies per 16S rRNA gene copies) in airplane sewage and the influent of wastewater treatment plants with connection to airports. Reported numbers are median values obtained by qPCR. Adjusted p-values refer to a two-sided Welch test.

Target class	ARG	Airplane (n=8)	WWTP, with airport (n=8)	adj. p-value
Colistin	<i>mcr1</i>	$2.8 \times 10^{-6}$	$9.7 \times 10^{-6}$	0.81
MLSB	<i>ermB</i>	$2.0 \times 10^{-2}$	$1.7 \times 10^{-2}$	1
$\beta$ -lactam	<i>bla</i> <sub>KPC-3</sub>	$1.6 \times 10^{-7}$	$2.0 \times 10^{-7}$	1
$\beta$ -lactam	<i>bla</i> <sub>OXA-48</sub>	$6.5 \times 10^{-6}$	$1.3 \times 10^{-3}$	0.33
$\beta$ -lactam	<i>bla</i> <sub>OXA-58</sub>	$1.6 \times 10^{-6}$	$6.1 \times 10^{-4}$	0.22
$\beta$ -lactam	<i>bla</i> <sub>TEM</sub>	$1.1 \times 10^{-3}$	$4.3 \times 10^{-4}$	1
$\beta$ -lactam	<i>mecA</i>	$2.7 \times 10^{-7}$	$2.3 \times 10^{-7}$	1
$\beta$ -lactam	<i>bla</i> <sub>CMY-2</sub>	$2.2 \times 10^{-4}$	$1.8 \times 10^{-5}$	0.81
$\beta$ -lactam	<i>bla</i> <sub>CTX-M-15</sub>	$3.2 \times 10^{-4}$	$4.5 \times 10^{-5}$	0.81
$\beta$ -lactam	<i>bla</i> <sub>CTX-M-32</sub>	$8.3 \times 10^{-4}$	$1.3 \times 10^{-4}$	0.81
$\beta$ -lactam	<i>bla</i> <sub>NDM-1</sub>	$1.5 \times 10^{-5}$	$2.2 \times 10^{-5}$	1
$\beta$ -lactam	<i>bla</i> <sub>VIM-2</sub>	0	$1.4 \times 10^{-4}$	0.037*
Sulphonamide	<i>sul1</i>	$4.7 \times 10^{-2}$	$9.2 \times 10^{-3}$	0.019*
Tetracyclin	<i>tetM</i>	$2.4 \times 10^{-2}$	$1.3 \times 10^{-3}$	9.4e-05***

### 3.4 Phenotypic resistance in *Escherichia coli*

The highest percentage of resistant *E. coli* isolates was generally detected in airplane samples (Table 3). The difference in proportions between airplane and WWTP samples was statistically significant for 18 out of 24 antibiotics. Some Odds ratios reached values around 40, namely for two cephalosporins (cefotaxime, cefuroxime) and one of the fluoroquinolones (ciprofloxacin). At the same time, the influent of treatment plants receiving sewage from airports did not exhibit elevated proportions of resistant *E. coli* when compared to treatment plants that do not receive such input (p-values generally  $> 0.15$ , results not shown).

Table 3: Antibiotic resistance among *E. coli* isolated from airplane sewage and the inlet of WWTP with connection to airports. Odds ratios (OR) greater than one indicate a higher level of resistance in the airplane samples compared to WWTP samples. p-values refer to Fisher’s exact test. SXT stands for the combination of trimethoprim and sulfamethoxazole also known as co-trimoxazole.

Antibiotic	Airplane	WWTP, with airport	OR	adj. p-value
Ampicillin	125:187	21:161	13.3	2.9e-24***
Amoxicillin-CA	65:187	8:161	10.1	1.3e-11***
Piperacillin	21:187	5:161	3.9	0.032*
Ticarcillin	55:187	4:161	16.3	1.3e-11***
Cefepime	7:187	0:161	Inf	0.099
Cefotaxime	97:187	4:161	41.9	1.2e-26***
Cefoxitin	9:187	1:161	8.1	0.12
Cefopodoxim	97:187	16:161	9.7	1.2e-16***
Ceftazidim	67:187	8:161	10.6	3.3e-12***
Cefuroxim	109:187	5:161	43.1	1.1e-30***
Doripenem	4:187	3:161	1.2	1
Ertapenem	4:187	2:161	1.7	1
Imipenem	83:187	7:161	17.4	3.5e-18***
Meropenem	56:186	7:161	9.4	7.8e-10***
Ciprofloxacin	94:187	4:161	39.3	2.2e-25***
Levofloxacin	82:187	8:161	14.8	4.8e-17***
Norfloxacin	31:187	10:161	3.0	0.024*
Amikacin	136:187	24:161	15.1	2.2e-27***
Gentamycin	64:187	10:161	7.8	4.1e-10***
Netilmicin	15:187	4:161	3.4	0.13
Tobramycin	126:187	17:161	17.3	1.2e-27***
Tigecyclin	4:187	1:161	3.5	1
Chloramphenicol	128:187	39:161	6.7	1.4e-15***
SXT	52:187	25:161	2.1	0.046*

Almost 90% of the *E. coli* isolates from airplane samples were resistant to at least one of the tested antibiotics (Fig. 5). This compares to about 45–60% in samples taken at WWTP. The peculiarity of airplane sewage is also reflected in the proportion of multi-resistant isolates. Airplane-borne *E. coli* were far more likely to be resistant against  $\geq 3$  classes of antibiotics than isolates obtained from raw sewage entering the WWTP (Fig. 5).

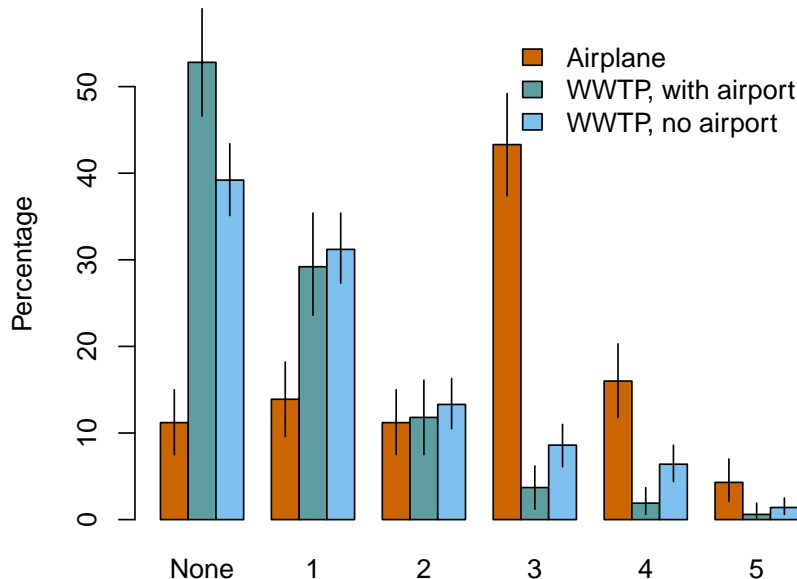


Figure 5: Prevalence of single- and multi-drug resistance among *E. coli* isolated from airplane sewage and WWTP influents with/without connection to airports. X-axis labels indicate the number of ineffective classes of drugs. The classes covered by susceptibility tests include penicillins, cephalosporines, carbapenemes, fluoroquinolones, aminoglycosides where each class is represented by 3–6 antibiotics (see 1<sup>st</sup> column of Table 3). Further drug classes represented by a single substance include tetracyclines, phenicols, and trimethoprim/sulfamethoxazole. Error bars represent 90% confidence intervals estimated by bootstrapping.

### 3.5 Methodological aspects

Since many of the available samples were processed in parallel through shotgun-sequencing and qPCR, there was a unique chance to compare the two approaches regarding the quantification of ARG. Doing so, we had to distinguish between unambiguous and potential hits in the metagenomes (Fig. 6). Unambiguous hits are those where a sample sequence ( $\approx 150$  bp) matched just a single ARG registered in the data base (black symbols). This is in contrast to potential hits (gray symbols), where the sample sequence matched multiple related ARG among which is the particular gene of interest.

In general, we observed a reasonable agreement between the metagenomics and qPCR data for ARG with relative abundances greater than about  $10^{-3}$  copies (16S rRNA gene copies)<sup>-1</sup>. The correlation coefficients were  $> 0.98$  for *su1* and *tetM*, for example, and  $> 0.93$  for *ermB* (based on unambiguous hits). Nevertheless, Fig. 6 also shows some characteristic mismatches between the two approaches. Most notably, there appears to be a negative bias in the metagenomics-based estimates. At relative abundances  $> 0.01$  the deviation hardly exceeds  $1/2$  log unit but underestimation gets stronger as relative abundances decline. That negative bias gradually turns into failure of the metagenomics approach (symbols accumulating on the lower axis) as the signatures of rare ARG occur just by chance in the set of sample sequences (about  $5 \times 10^7$  in this study).

However, not all of the deviations depicted in Fig. 6 should be blamed on the limitations of metagenomics. For example, symbols associated with *bla*<sub>OXA-48</sub> and *bla*<sub>TEM</sub> are far off the 1:1 line. This might well be due to unspecific amplification of primers

306 resulting in an overestimation of ARG abundances by qPCR.

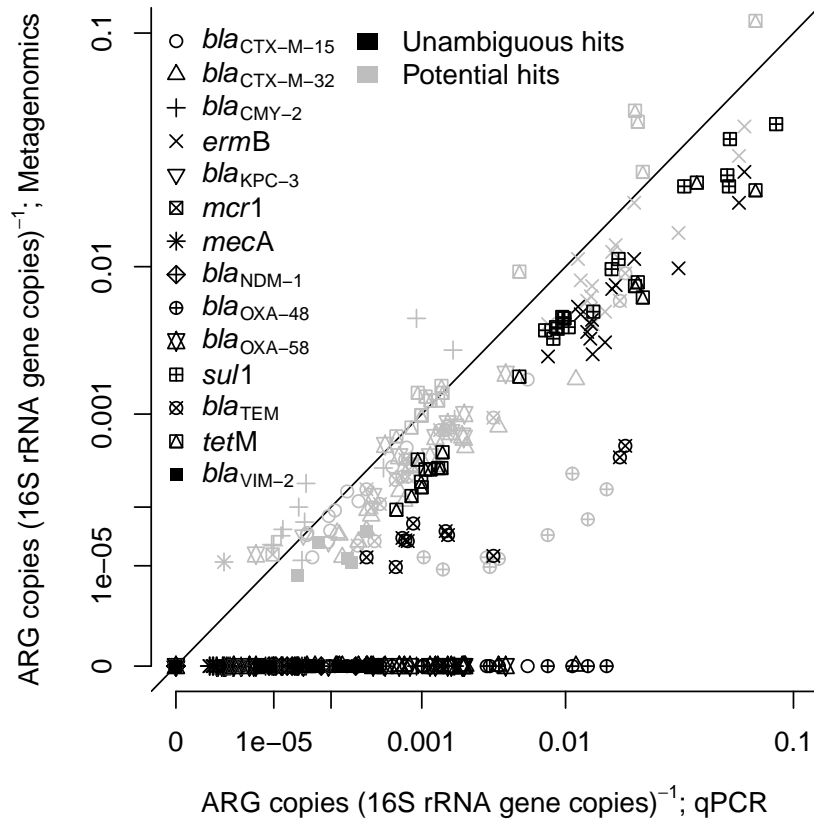


Figure 6: Comparison of relative ARG abundances obtained with qPCR and metagenomics for identical extracts of DNA. The diagonal indicates a 1:1 match. The data include raw sewage samples from all sources (airplane and WWTP). Note the custom axes resulting from square-root transformation.

## 307 4 Discussion

308 Cultivation and culture-independent techniques yielded a largely consistent and well  
 309 differentiated picture of the prevalence of ARB/ARG in untreated wastewater of different  
 310 origins. In agreement with initial expectations, sewage from airplane tanks was found to  
 311 stand out from conventional sewage in terms of ARG diversity (Fig. 2). Nevertheless, the  
 312 rarefaction models plotted in Fig. 3 suggest that the difference between airplane sewage  
 313 and untreated municipal wastewater in terms of ARG diversity might level off for very  
 314 large numbers of analyzed sequences. Considering the fact that municipal wastewater  
 315 integrates bacteria from various sources besides those related to human waste (e.g. from  
 316 pets, slaughterhouses, soil runoff, etc.) a convergence of the curves seems plausible. Thus,  
 317 the main feature of airplane sewage appears to be the fact that a large number of different  
 318 ARG can already be found in limited quantities of bacterial DNA. The mixing of human  
 319 gut bacteria from different geographical backgrounds (hypothesis 1) provides a plausible  
 320 explanation for this finding. Considerable variation in human resistomes is known to  
 321 exist already at European level ([European Centre for Disease Prevention and Control, 2017](#);  
 322 [Pärnänen et al., 2019](#)) and even larger disparities are expected on global scales. For  
 323 example, [Bengtsson-Palme et al. \(2015a\)](#) demonstrated the import of ESBL-producing  
 324 *E. coli* by travelers returning from India while no such import was observed from Africa.  
 325 However, the elevated diversity of ARG in airplane sewage could also be due to alternative

mechanisms. For example, disparities in ARG diversity between samples of different origin might – at least in parts – reflect contrasts in taxonomic diversity. In particular, the elevated number of unique ARG in airplane sewage coincided with an increased diversity of bacterial groups as reflected in taxonomic evenness (Fig. 1) as well as in the number of bacterial families detected per 10<sup>5</sup> DNA sequences (Sec. 3.1). Against this background, the true cause(s) of the increased ARG diversity in airplane-borne sewage are yet to be explored. Instead of focusing on ARG diversity, future research might also attempt to identify specific ARG which are characteristic for wastewaters of different origin. Our metagenomics-based data suggest that such characteristic genes exist. For example, the beta-lactamase gene *bla*<sub>CARB-4</sub> was found in high relative abundances in airplane sewage while it was never detected in any sample of municipal wastewater without contribution from airports. Similarly, the carbapenemase resistance gene *bla*<sub>OXA-427</sub> was frequently detected in samples from WWTP influents while its signature was not found in any sample of airplane sewage.

Our data clearly support the second hypothesis according to which airplane-borne sewage stands out from common untreated municipal wastewater in terms of the relative abundance of ARG. The elevated prevalence of antibiotic resistance in airplane sewage was most clearly demonstrated by drug susceptibility tests carried out on *E. coli* (Table 3, Fig. 5). The high abundance of multi-resistant *E. coli* in airplane sewage is particularly remarkable. For example, the proportion of isolates carrying a combined resistance against 3<sup>rd</sup> generation cephalosporins, fluoroquinolones and aminoglycosides was increased by a factor of about eight compared to German clinical isolates (28.9% compared to 3.5%; [European Centre for Disease Prevention and Control, 2017](#)). With regard to the prevalence of genotypic resistance, the clearest differentiation between wastewaters of different origin was obtained by the metagenomics approach (Fig. 4). The median relative abundance of ARG was higher in airplane-borne sewage than in conventional sewage for seven out of nine classes of target drugs. Similar to the case of gene diversity, the elevated prevalence of ARG/ARB in airplane sewage might reflect differences in gut microbiomes between the local population (Germany) and the flights' or passengers' countries of origin. However, storage conditions in the airplanes' wastewater tanks provide an alternative explanation. Those tanks typically contain disinfectants. A common formulation certified for use in aircraft toilets lists alkylbenzyltrimethylammonium chloride, a quaternary ammonium compound, as its major ingredient. A number of potential linkages between this compound and antibiotic resistance bacteria is known to exist (see [SCENIHR, 2009](#), Sec. 3.8.5). The sewage tanks might thus serve as incubators that select for antibiotic resistance via mechanisms of, e.g., cross- or co-resistance ([Buffet-Bataillon et al., 2012](#)). The "breeding" of ARB should be particularly efficient if the tanks are not completely purged, leaving a highly resistant inoculum for continued vertical and horizontal ARG transfer.

In order to assess the potential risk associated with the special resistome of airplane sewage, information on the fate of the respective ARG/ARB is required. Our data indicate that airplane-borne sewage is strongly "diluted" upon mixing with wastewater from other sources. None of the employed methods (susceptibility tests, qPCR, metagenomics) indicated significant shifts in ARG diversity or ARG/ARB prevalence between samples of raw sewage acquired from WWTP with and without connection to airports. Assuming that bacteria and genes do not undergo considerable retention or degradation in the sewer system, that lack of significance should mainly reflect the limits of current analytical methods to detect small increments in ARG/ARB abundances in the presence of considerable background levels (unfavorable signal-to-noise ratio).

One might be tempted to conclude that airport-borne sewage is of little relevance for the dissemination of ARG because of the apparently strong dilution. Furthermore,

airplane tanks are just one source of ARG and other hot-spots of antibiotic resistance are known to exist, e. g. large health care facilities. Such reasoning, however, disregards an essential property of genetic material, namely the potential for replication via horizontal and vertical transfer. Consequently, even very small amounts of ARG released into the water cycle may spread within the aquatic environment with the chance of (re)emerging in human pathogens. From this point of view, the potential threat coming from airport-borne sewage is not the quantity of imported ARG but the dissemination of resistances which are rarely found in the local environmental systems. This relates not only to rare ARG but also to combined resistances against multiple classes of drugs. With the example of *E. coli*, airplane-sewage was shown to be an unusual source of highly multi-resistant isolates. Nevertheless, we currently cannot provide evidence for the fact that rare ARG and/or combined resistances originating from airplane-sewage actually undergo considerable enrichment via selection or horizontal gene transfer in the receiving treatment plants. As pointed out by [Bengtsson-Palme et al. \(2016\)](#), comprehensive analyses of resistant and susceptible strains in WWTP influents and effluents would be necessary to specifically target that question. As a consequence of our study, we suggest to first explore evolutionary processes inside airplane waste tanks and to look for options that prevent (or deal with) the increased level of antibiotic resistance "begin-of-pipe".

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## Competing interests

The authors declare no competing interests.

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Table S.1: Characteristics of individual samples. Location names were substituted by anonymous integer codes. Volumes represent the amount of sample from which DNA was extracted.

Date	City code	Origin	Volume (mL)	DNA (ng $\mu\text{L}^{-1}$ )	260/280 nm ratio	260/230 nm ratio	<i>E. coli</i> isolates	Sequen- ced	qPCR done	pH	EC (mS $\text{cm}^{-1}$ )	16S gene copies $\text{mL}^{-1}$
2016-10-31	5	Airplane	10	109	1.875	1.025	0	✓	✓	9.0	9.44	6.8E+08
2016-11-14	1	Airplane	25	771	1.91	2.095	46	✓	✓	8.3	10.62	2.2E+09
2016-11-17	3	Airplane 1	17.5	322	1.885	1.84	48	✓	✓	9.0	13.00	2.2E+09
2016-11-17	3	Airplane 2	10	260	1.89	1.56	47	✓	✓	8.9	10.16	3.0E+09
2016-11-29	4	Airplane	20	136	1.86	1.27	24	✓	✓	8.9	8.23	8.5E+08
2016-12-05	5	Airplane	20	81	1.855	0.725	0	✓	✓	9.2	14.09	4.0E+08
2016-12-12	1	Airplane	25	183	1.88	1.555	22	✓	✓	8.7	4.24	8.3E+08
2017-01-31	2	Airplane	10	221	1.92	2.19	0	✓	✓	9.0	20.83	2.9E+09
2016-11-29	4	WWTP, with airport, sewer 1	65	356	1.87	1.69	23			7.8	1.85	5.6E+08
2016-11-29	4	WWTP, with airport, sewer 2	70	251	1.92	1.735	24	✓	✓	7.4	1.76	3.6E+08
2017-01-17	5	WWTP, with airport	100	185	1.96	1.69	23	✓	✓	6.6	2.21	9.7E+07
2017-01-31	2	WWTP, with airport	100	536	1.98	1.97	0			8.2	2.00	3.8E+08
2017-02-14	4	WWTP, with airport	50	88	2.17	0.2	0	✓	✓	7.5	2.35	5.5E+07
2017-02-28	3	WWTP, with airport	100	175	2.025	1.375	24	✓	✓	7.4	1.62	1.3E+08
2017-03-06	3	WWTP, with airport	100	434	1.95	1.75	24	✓	✓	6.8	0.76	2.1E+08
2017-03-14	9	WWTP, with airport	50	180	1.93	1.605	24	✓	✓	6.6	1.34	3.1E+08
2017-03-15	5	WWTP, with airport	100	222	1.9	1.55	0	✓	✓	6.9	1.26	1.8E+08
2017-05-25	8	WWTP, with airport	100	587	1.97	1.09	19			8.0	NA	2.6E+08
2016-09-17	1	WWTP, no airport	100	380	1.92	1.715	0	✓	✓	7.7	0.54	2.9E+08
2016-12-06	1	WWTP, no airport	100	291	1.89	1.78	21	✓	✓	7.0	1.81	3.0E+08
2017-01-03	1	WWTP, no airport	100	180	1.99	1.25	23	✓	✓	7.4	1.88	7.4E+07
2017-02-01	1	WWTP, no airport	100	310	1.99	1.51	0	✓	✓	7.7	2.01	2.1E+08
2017-02-24	1	WWTP, no airport	100	304	2.395	1.915	24	✓	✓	7.4	0.87	1.9E+08
2017-02-28	1	WWTP, no airport	100	369	1.985	1.79	0			7.3	1.43	1.5E+08
2017-03-07	1	WWTP, no airport	100	544	1.915	1.835	24	✓	✓	6.7	1.08	3.1E+08
2017-03-06	3	WWTP, no airport	100	567	1.955	1.74	24	✓	✓	6.7	0.73	2.8E+08
2017-03-14	9	WWTP, no airport	50	212	1.97	1.155	24	✓	✓	6.7	1.37	3.4E+08
2017-05-12	1	WWTP, no airport	100	372	1.92	1.035	0	✓	✓	6.9	1.05	2.4E+08
2017-05-25	8	WWTP, no airport, sewer 1	100	411	1.875	0.97	24	✓	✓	7.9	NA	2.0E+08

Table S.1, continued

2017-05-25	8	WWTP, no airport, sewer 2	100	453	1.895	1.86	22	✓	7.7	NA	2.9E+08
2017-05-25	8	WWTP, no airport, sewer 3	100	125	1.805	0.495	12	✓	8.0	NA	5.5E+07
2017-05-25	8	WWTP, no airport, sewer 4	100	393	1.855	1.295	23	✓	7.7	NA	1.6E+08
2017-07-11	6	WWTP, no airport	100	111	1.87	1.195	22	✓	NA	NA	8.4E+07
2017-07-27	1	WWTP, no airport	100	359	1.915	1.785	23	✓	7.4	0.82	2.6E+08
2017-08-02	1	WWTP, no airport	100	281	1.905	1.675	24	✓	NA	0.85	2.2E+08
2017-08-10	1	WWTP, no airport	100	56	1.945	0.495	24	✓	7.3	0.49	3.1E+07
2017-08-10	7	WWTP, no airport	100	91	1.96	0.68	24	✓	7.3	0.89	8.3E+07
2017-08-31	6	WWTP, no airport	100	362	1.885	1.68	24	✓	7.2	0.79	9.4E+08
2017-11-05	7	WWTP, no airport	100	365	1.89	1.68	0	✓	NA	NA	2.6E+08
2018-01-04	6	WWTP, no airport	100	347	1.94	1.98	0	✓	NA	NA	1.4E+09

Table S.2: List of qPCR primers.

Gene		Primer sequence	Amplicon size (bp)	Reference
<i>bla</i> <sub>TEM</sub>	Fwd.	TTCCTGTTTTTGCTCACCCAG	112	NORMAN Network <sup>1</sup>
	Rev.	CTCAAGGATCTTACCGCTGTTG		
<i>ermB</i>	Fwd.	TGAATCGAGACTTGAGTGTGCAA	71	Alexander et al. (2016)
	Rev.	GGATTCTACAAGCGTACCTT		
<i>tetM</i>	Fwd.	GGTTTCTCTTGATACTTAAATCAATCR	88	Peak et al. (2007)
	Rev.	CCAACCATAYAATCCTTGTTTCRC		
<i>sul1</i>	Fwd.	CGCACCGGAAACATCGCTGCAC	161	NORMAN Network <sup>1</sup>
	Rev.	TGAAGTTCGCCGCAAGGCTCG		
<i>bla</i> <sub>CMY-2</sub>	Fwd.	CGTTAATCGCACCATCACC	172	Kurpiel and Hanson (2012)
	Rev.	CGTCTTACTAACCGATCCTAGC		
<i>bla</i> <sub>CTX-M-15</sub>	Fwd.	CTATGGCACCACCAACGATACTYM	103	ANSWER Network <sup>2</sup>
	Rev.	ACGGCTTTCTGCCTTAGGTT		
<i>bla</i> <sub>CTX-M-32</sub>	Fwd.	CGTCACGCTGTTGTTAGGAA	155	NORMAN Network <sup>1</sup>
	Rev.	CGCTCATCAGCAGGATAAAG		
<i>bla</i> <sub>OXA-48</sub>	Fwd.	TGTTTTTGGTGGCATCGAT	177	Monteiro et al. (2012)
	Rev.	GTAAMRATGCTTGGTTCGC		
<i>bla</i> <sub>OXA-58</sub>	Fwd.	GCAATTGCCTTTTAAACCTGA	152	Szczepanowski et al. (2009)
	Rev.	CTGCCTTTTCAACAAAACCC		
<i>mecA</i>	Fwd.	CGAACGTTCAATTTAATTTTGTTAA	91	Volkmann et al. (2004)
	Rev.	TGGTCTTTCTGCATTCTGGA		
<i>bla</i> <sub>NDM-1</sub>	Fwd.	TTGGCCTTGCTGTCCTTG	82	Monteiro et al. (2012)
	Rev.	ACACCAGTGACAATATCACCG		
<i>bla</i> <sub>VIM-2</sub>	Fwd.	GAGATTCCCACGCACTCTCTAGA	93	van der Zee et al. (2014)
	Rev.	AATGCGCAGCACCAGGATAG		
	probe	ACGCAGTGCCTTCGGTCCAGT		
<i>bla</i> <sub>KPC-3</sub>	Fwd.	CAGCTCATTCAAGGGCTTTC	196	Szczepanowski et al. (2009)
	Rev.	GGCGGCGTTATCACTGTATT		
<i>mcr1</i>	Fwd.	GGGCCTGCGTATTTAAGCG	183	Hembach et al. (2017)
	Rev.	CATAGGCATTGCTGTGCGTC		
16S rRNA	Fwd.	TCCTACGGGAGGCAGCAGT	195	NORMAN Network <sup>1</sup>
	Rev.	ATTACCGCGCTGCTGG		

<sup>1</sup>[www.norman-network.net](http://www.norman-network.net)<sup>2</sup>[www.answer-itn.eu](http://www.answer-itn.eu)